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Biochemical Risk Factors in Angiographically Established Stenosis of Cerebral Arteries

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Aim. To determine the significance of the association between biochemical risk factors for cerebrovascular atherosclerosis (lipid parameters, lipoprotein(a), total homocysteine, total antioxidant status, trace elements, and electrolytes) and the degree of stenosis of cerebral arteries scored by digital subtraction angiography.

Methods. The study included 35 patients with angiographically established < 50% stenosis of cerebral arteries and 55 patients with ≥50% stenosis of cerebral arteries, including obliteration. The control group consisted of 51 patients with normal cerebral arteries on ultrasound examination. Biochemical parameters were measured in all participants according to the standard laboratory protocols.

Results. Logistic regression analysis showed two independent and significant biochemical risk factors associated with the severity of cerebrovascular stenosis: lipoprotein(a) for patients with different degrees of stenosis, and total antioxidant status for patients with severe stenosis of more than 50%. Univariate statistical evaluation showed significantly higher homocysteine levels in the group of patients with ≥50% stenosis than in the control group (median 14.84 μmol/L vs median 12.40 μmol/L, $p < 0.05$).

Conclusion. Increased lipoprotein(a) and low total antioxidant status values seem to be the most significant independent biochemical risk factors for the development of cerebrovascular stenosis. Mild hyperhomocysteinemia seems to be an additional discriminating indicator of the severe cerebrovascular stenosis. These factors may be useful for early identification and recognition of patients with cerebrovascular atherosclerosis.

Key words: *cerebral arteries; constriction, pathologic; homocysteine; lipoprotein(a); risk factors*

The development of cerebrovascular insufficiency is influenced by a number of biochemical and other potential risk factors, such as genetic determinants, demographic and lifestyle characteristics (1). Recent studies have recognized the association of total homocysteine and lipoprotein(a) concentrations and total antioxidant capacity with a greater risk for premature development of atherosclerosis, but there has been little data on the association between the biochemical risk factors and severity of cerebrovascular disease (2-4).

Serum lipoprotein(a) has been considered an atherosclerotic risk factor under considerable genetic control but independent of all other biochemical and exogenous modifications (5). It shows size polymorphism, with up to 34 different molecular mass lipoprotein(a) isoforms representing the serum lipoprotein(a) (2,6).

Moderate serum hyperhomocysteinemia could be the result of a nutritional deficiency of folic acid and vitamin B_{12}; heterozygote enzyme deficiencies, such as deficiency of cystathione ß-synthase or 5,10-methylenetetrahydrofolate reductase; and demographic and lifestyle influences (7). Only 1-2% of total homocysteine circulates in the blood freely in a reduced form, whereas even up to 99% is readily oxidized and mostly protein-bound. The induction of the atherogenic process by hyperhomocysteinemia seems to be associated with an accelerated formation of the reactive oxygen species, enhanced low-density lipoprotein oxidation, increased incorporation of lipoprotein(a) into fibrin, and change of hemostatic conditions toward procoagulant effects on endothelial cells (3,8). The oxidation hypothesis of atherosclerotic disease emphasizes that free radicals generation and consequent oxidative stress have a distinctive role in the pathogenesis of atherosclerotic processes (4,9,10). For this reason, values of total antioxidant status are frequently used in clinical work as valuable biochemical markers of the total antioxidant capacity. Mineral imbalance in arterial wall and serum was reported as one of the etiological factors in the progres-

sion of atherosclerosis (11). Damage of endothelial walls may cause the release of protein-bound metal ions, which can potentiate damage by catalyzing the decomposition of hydroperoxides and formation of free radicals (12). Subsequent studies showed that serum concentrations of trace elements (copper, zinc) and electrolytes (total calcium and magnesium) are changed in different atherosclerotic diseases (11,13, 14).

The aim of this study was to explore the association between the biochemical risk factors for cerebrovascular atherosclerosis, including lipid parameters, lipoprotein(a), total homocysteine, total antioxidant status, trace elements (copper, zinc), and electrolytes (total calcium and magnesium), and different degrees of stenosis of cerebral arteries scored by the method of digital subtraction angiography, compared with the control group of participants with normal cerebral arteries presented at the ultrasound examination.

Patients and Methods

Patients

The study group included 141 patients, 52 women and 89 men, with symptoms of the cerebrovascular insufficiency. The presence of coronary atherosclerosis was excluded by physical and electrocardiographic examination. Before angiography, duplex ultrasound examinations of cerebral arteries were performed on all patients at the Institute of Diagnostic and Interventional Radiology, Merkur University Hospital. Patients with a negative ultrasound examination of cerebral arteries were allocated to the control group. The control group consisted of 51 patients, 20 women (median age, 57 years; range, 30-75) and 31 men (median age, 57 years; range, 30-71). All patients with a positive ultrasound finding indicating stenosis of different degree were referred to angiography of cerebral vessels according to the standard procedure. Common carotid artery, internal carotid artery, and its intracranial branches were examined. The degree of stenosis at angiography was measured according to a standard method (15). Based on the angiographic findings, the patients with cerebral atherosclerosis were divided into two groups. The first group consisted of 35 patients, 13 women (median age, 65 years; range, 40-75) and 22 men (median age, 60 years; range, 30-80) with a moderate degree of stenosis, ie, $<50\%$ of the arterial lumen. The second study group included 55 patients, 19 women (median age, 62 years; range, 34-74) and 36 men (median age, 63 years; range, 43-78), with 50-99% stenosis or obliteration of cerebral arteries. Participants reporting daily smoking were defined as smokers. Obesity was defined in terms of the patient's body mass index calculated as weight in kg/height in m^2 (16). The patients with the body mass index ≥ 25 were considered overweight.

Serum Samples

For biochemical analysis, blood samples were drawn from all participants after overnight fasting from the cubital vein. Serum samples were separated from blood cells within 30 min to prevent the passage of homocysteine from red cells into the serum. All biochemical constituents of sera except lipoprotein(a) were determined on fresh sera on the day of blood collection. Sera for the determination of lipoprotein(a) were frozen at -70°C and analyzed within 2 months, as the storage of serum in this condition does not change the lipoprotein concentration (17).

Biochemical Analysis

Serum total cholesterol and triacylglycerol were measured by methods routinely used in medical-biochemical laboratories (enzymatic PAP-method) on the Olympus AU 600 Analyser (Olympus Mishima Co., Shizuoka, Japan). High-density lipoprotein cholesterol (HDL-C) was measured with a direct method based on selective inhibition of the non-HDL fractions by means of polyanions. We calculated low-density lipoprotein cholesterol (LDL-C) using Friedewald formula. The index of atherosclerosis and the established risk factor were calculated as LDL-C/HDL-C and triacylglycerol/HDL-C. Lipoprotein(a) was quantified with an enzyme linked immunosorbent assay plaque technique (Apo-Tek lipoprotein(a), Immuno AG, Vienna, Austria). Total homocysteine in serum was estimated with a fluorescence polarization immunoassay technique as a fully automated method on an IMX Analyser (Abbott Laboratories Diagnostic Division, Abbott Park, IL, USA). Serum total antioxidant status was measured with the Randox enzyme-chromogen assay (Cat. No. NX2332, Randox Laboratories Ltd., Crumlin, UK) applied on an Olympus AU-600 Analyzer, which enables the assessment of the integrated antioxidant system. Copper, zinc, and total calcium and magnesium were measured by flame atomic absorption spectroscopy on the Pye Unicam SP9 Analyzer (Philips Ltd., Cambridge, England).

The Mann-Whitney U-test was applied to evaluate the differences between the groups, with $p<0.05$ considered statistically significant. Chi-square test was used to analyze qualitative variables (sex and smoking habits). Logistic regression analysis was performed with SAS software (Proc logistic program, Version 6, SAS Institute Inc., Cary, NC, USA, 1998) to evaluate simultaneously the relationship of biochemical and demographic factors in the groups of patients with different degrees of stenosis vs control group. Lipoprotein(a) was transformed to a binary variable, with a cut-off level at 0.3 g/L.

Results

The control group and the groups of patients with different degrees of cerebrovascular stenosis were well matched in terms of demographic and lifestyle characteristics (age, body mass index, and blood pressure) (Table 1). No significant differences were found between the two subgroups with cerebrovascular stenosis in sex distribution (Yates corrected chi-square = 0.001, $p=0.975$ in the group with $<50\%$ of stenosis; Yates corrected chi-square = 0.088, $p=0.767$ in the group with $\geq 50\%$ of stenosis) or smoking habits (Yates corrected chi-square = 1.730, $p=0.188$ in the group with $<50\%$ of stenosis; Yates corrected chi-square = 0.002, $p=0.965$ in the group with $\geq 50\%$ of stenosis). The proportion of daily smokers was 40.0% in the group of patients with $<50\%$ stenosis, 54.5% in the group of patients with $\geq 50\%$ of stenosis, and 56.8 % in control no-stenosis group. The mean values of the body mass index in all groups were <25 kg/m^2, indicating overweight. The systolic and diastolic blood pressures did not differ significantly across the groups, whereas the influence of blood pressure on the obtained results was not considered relevant.

The patients with cerebrovascular stenosis and control group did not differ in their lipid profiles (triacylglycerol, total cholesterol, HDL-C, LDL-C, index of atherosclerosis, and established risk factor ratios) (Table 1). However, in both control and patients groups the median values for triacylglycerol, total cholesterol, LDL-C, index of atherosclerosis, and established risk factor ratios were higher and for HDL-C lower than the recommended values for the prevention of atherosclerosis (18) (Table 2).

Lipoprotein(a) values were significantly higher in patients with cerebrovascular stenosis than in the control group (median 0.32 g/L in the group of patients with $<50\%$ stenosis, and 0.33 g/L in the group of patients with $\geq 50\%$ stenosis vs 0.11 g/L in the control group; $p<0.05$) (Table 1).

Table 1. Demographic data and biochemical parameters (median, range) of patients with <50% and ≥50% of cerebrovascular stenosis[a]

Parameter	Control no-stenosis group	Patients with cerebrovascular stenosis			
		<50% of stenosis	p[a]	≥50% of stenosis	p[b]
No. of patients	51	35		55	
Age (years)	57 (30-75)	60 (30-80)	0.484	63 (34-78)	0.260
Body mass index (kg/m2)	25.4 (18.5-33.9)	25.9 (17.7-41.3)	0.864	25.3 (18.3-33.3)	0.642
Diastolic blood pressure (kPa)	11.9 (6.6-15.9)	11.9 (8.6-14.6)	0.632	10.6 (8.6-14.6)	0.068
Systolic blood pressure (kPa)	21.3 (14.2-26.6)	19.9 (14.6-25.3)	0.515	21.3 (15.3-27.3)	0.391
Total cholesterol (mmol/L)	6.0 (2.2-9.4)	6.1 (4.6-9.0)	0.251	6.0 (3.8-9.4)	0.606
Triacylglycerol (mmol/L)	1.90 (0.70-6.60)	1.7 (0.7-6.3)	0.361	1.9 (0.6-4.4)	0.292
HDL-C (mmol/L)	1.0 (0.6-1.5)	1.0 (0.6-1.8)	0.639	1.1 (0.4-2.1)	0.348
LDL-C (mmol/L)	3.7 (1.0-7.3)	4.2 (1.4-7.1)	0.053	4.0 (2.1-7.1)	0.333
LDL-C/HDL-C	3.6 (1.5-6.8)	4.6 (2.3-8.8)	0.011	3.8 (1.3-13.7)	0.489
Total cholesterol /HDL-C	5.7 (2.7-9.6)	6.5 (3.6-11.1)	0.057	5.8 (1.8-18.5)	0.904
Lipoprotein(a) (g/L)	0.11 (0.01-0.43)	0.32 (0.01-0.67)	0.000	0.33 (0.01-0.59)	0.000
Homocysteine (μmol/L)	12.40 (5.28-25.10)	11.65 (7.44-32.30)	0.623	14.84 (10.03-49.95)	0.010
TAS (mmol/L)	1.55 (1.39-1.97)	1.49 (1.22 -3.46)	0.013	1.40 (1.20-1.69)	0.000
Copper (μmol/L)	19.4 (13.0-30.5)	20.0 (14.2-31.2)	0.083	19.9 (13.1-26.8)	0.131
Zinc (μmol/L)	13.0 (8.7-29.1)	13.0 (10.9-24.7)	0.515	12.4 (9.9-18.8)	0.244
Total calcium (mmol/L)	2.32 (2.08-2.63)	2.33 (1.68-2.56)	0.748	2.35 (2.04-2.70)	0.932
Total magnesium (mmol/L)	0.82 (0.59-1.00)	0.85 (0.72-1.08)	0.036	0.84 (0.54-1.16)	0.535

[a]Abbreviations: HLD-C – high density lipoprotein cholesterol; LDL-C – low density lipoprotein cholesterol; TAS – total antioxidant status.
[b]vs control group, Mann-Whitney test.

Logistic regression analysis showed that lipoprotein(a) was independently associated with different degrees of cerebrovascular stenosis (Tables 3 and 4).

Significantly higher homocysteine values were found in the group of patients with ≥50% stenosis, compared with the control group (median 14.84 μmol/L vs 12.40 μmol/L in the control group; $p < 0.05$) (Table 1), whereas logistic regression analysis revealed that mild hyperhomocysteinemia was not a significant independent indicator of cerebrovascular stenosis (Tables 3 and 4). The median values found in both controls and two groups of patients with different degrees of cerebrovascular stenosis were within the population-based reference intervals for the adult healthy population (19) (Table 2).

Total antioxidant status values were lower in patients with the cerebrovascular stenosis than in the control group (median 1.49 mmol/L in the group with <50% stenosis and 1.40 mmol/L in the group with

Table 2. Biological variation of biochemical parameters[a,b]

Parameter	Biological variations		Recommended values and health-associated reference intervals
	intraindividual	interindividual	
Total cholesterol (mmol/L)	6.0	15.2	<5.2
Triacylglycerol (mmol/L)	21.0	37.2	men <1.8
			women <1.5
HDL-C (mmol/L)	7.1	19.7	men >1.4
			women >1.7
LDL-C (mmol/L)	6.6	14.7	<3.9
Lipoprotein(a) (g/L)	8.5	85.8	<0.3
Homocysteine (μmol/L)	9.4	23.9	men 5.9-15.3
			women 4.9-11.6
TAS (mmol/L)	2.8	4.5	1.30-1.77
Copper (μmol/L)	4.9	13.6	12.2-25.1
Zinc (μmol/L)	9.3	9.4	9.9-17.9
Total calcium (mmol/L)	1.9	2.8	2.14-2.53
Total magnesium (mmol/L)	3.6	6.4	0.65-1.05

[a]According to ref. 19.
[b]Abbreviations: HLD-C – high density lipoprotein cholesterol; LDL-C – low density lipoprotein cholesterol; TAS – total antioxidant status.

Table 3. Logistic regression analysis of predictive variables for cerebrovascular stenosis in patients with <50% of cerebrovascular stenosis (n = 35) compared with the control group (n = 50)[a]

Predictive variables	Odds ratio (95% CI)	p[b]
Sex	1.606 (0.350-7.359)	0.542
Smokers	0.493 (0.143-1.701)	0.263
Age (years)	1.029 (0.963-1.099)	0.403
Body mass index (kg/m2)	1.019 (0.887-1.170)	0.791
Total cholesterol (mmol/L)	2.748 (0.846-8.926)	0.093
Triacylglycerol (mmol/L)	0.689 (0.375-1.267)	0.231
HDL-C (mmol/L)	0.357 (0.013-9.796)	0.542
LDL-C (mmol/L)	0.508 (0.172-1.506)	0.222
Lipoprotein(a) (g/L)	16.941 (3.931-73.012)	<0.001
Homocysteine (μmol/L)	0.994 (0.878-1.125)	0.926
TAS (mmol/L)	0.114 (0.010-1.288)	0.079
Copper (μmol/L)	1.242 (1.024-1.505)	0.027
Zinc (μmol/L)	1.331 (1.049-1.688)	0.018
Total calcium (mmol/L)	0.041 (0.002-9.836)	0.253
Total magnesium (mmol/L)	2.1×10^5 ($0.074\text{-}6.1 \times 10^5$)	0.187

[a]Abbreviations: HLD-C – high density lipoprotein cholesterol; LDL-C – low density lipoprotein cholesterol; TAS – total antioxidant status.
[b]Wald chi-square test.

Table 4. Logistic regression analysis of predictive variables for cerebrovascular stenosis in patients with ≥50% of cerebrovascular stenosis (n = 55) compared with control group (n = 50)[a]

Predictive variables	Odds ratio (95% CI)	p[b]
Sex	1.717 (0.450-6.543)	0.429
Smokers	1.155 (0.328-4.065)	0.822
Age (years)	1.053 (0.978-1.135)	0.171
Body mass index (kg/m2)	1.000 (0.826-1.211)	1.000
Total cholesterol (mmol/L)	2.825 (0.272-29.303)	0.384
Triacylglicerol (mmol/L)	0.617 (0.188-2.017)	0.424
HDL-C (mmol/L)	3.356 (0.122-92.567)	0.474
LDL-C (mmol/L)	0.492 (0.048-5.021)	0.549
Lipoprotein(a) (g/L)	5.922 (1.473-23.816)	0.012
Homocysteine (μmol/L)	1.167 (0.981-1.388)	0.082
TAS (mmol/L)	<0.001 (both limits <0.001)	<0.001
Copper (μmol/L)	1.044 (0.860-1.267)	0.660
Zinc (μmol/L)	1.159 (0.870-1.542)	0.314
Total calcium (mmol/L)	14.039 (0.055-3554.767)	0.350
Total magnesium (mmol/L)	0.007 (<0.001-6.478)	0.155

[a]Abbreviations: HLD-C – high density lipoprotein cholesterol; LDL-C – low density lipoprotein cholesterol; TAS – total antioxidant status.
[b]Chi-square test.

≥50% stenosis vs 1.55 mmol/L in the control group; $p < 0.05$) (Table 1). Logistic regression analysis showed a strong association between the high degree of cerebrovascular stenosis (≥50%) and decreased level of total antioxidant status concentrations (Table 4). Median values of total antioxidant status were within the population-based reference intervals in all studied groups (12) (Table 2).

There was no significant association between the total calcium and magnesium concentrations in the patients with different degree of cerebrovascular stenosis vs control group (Tables 1, 3, and 4) and reference intervals of the healthy population (20) (Table 2). Logistic regression analysis showed a strong association between the low degree of cerebrovascular stenosis and increased concentrations of trace elements (copper and zinc) (Table 4).

Discussion

Significant changes associated with atherosclerosis could be the result of the lifestyle and demographic characteristics based on genetic determinants and a number of biochemical and physiological risk factors. All examined groups in this study were characterized by high frequency of cigarette smoking and overweight, which could be considered as possible risk factors contributing to the increased risk of cerebrovascular stenosis.

Today, it is generally accepted that dyslipidemia is one of the major biochemical risk factors for the development of atherosclerotic disease (1,18,21,22). In our study, median values for total cholesterol, LDL-C, triacylglycerols, index of atherosclerosis, and established risk factor ratios were higher and for HDL-C lower than the recommended values for the prevention of atherosclerotic disease (18) or in healthy adult population in Croatia (23). This indicated a possible contribution of dyslipidemia to the risk of developing atherosclerotic disease. The levels of total cholesterol, LDL-C, HDL-C, triacylglycerols, index of atherosclerosis, and established risk factor ratios in our study were not the discriminating indicators of the degree of cerebrovascular stenosis, which is consistent with findings of previous studies (3).

The recent data on biological variation of lipoprotein(a) in adults (9) and in the healthy population of school children and adolescents in Croatia (24) showed that lipoprotein(a) distribution in the healthy population is highly skewed toward the lower concentrations, with the cut-off value at < 0.3 g/L, intraindividual variation of 8.5%, and wide interindividual variations even up to 86% (25). Our results showed that lipoprotein(a) concentrations in patients with cerebrovascular stenosis were significantly increased compared with those in the control group and health-associated reference intervals. These findings confirmed lipoprotein(a) as the only factor significantly associated with stenosis of cerebral arteries of different degree (2). This is in agreement with previous studies indicating that a high lipoprotein(a) level is an independent predictor of the atherosclerotic disease (2,6).

Total homocysteine analysis has become a part of the risk assessment profile of patients with premature atherosclerotic disease (19). Data on biological variation (9), showing high individuality and a wide range of population-based reference intervals (19), favor the adoption of cut-off points based upon relative risk for development of the atherosclerotic disease for the interpretation of individual result. Values between 11.7 and 16.0 μmol/L are considered as mild hyperhomocysteinemia (26), although population-based reference ranges indicated 15.3 μmol/L as the upper reference limit for men and 11.6 μmol/L for women (19). It has been suggested that homocysteine values < 12 μmol/L were optimal, although current evidence suggests that target level for homocysteine should be < 9.0 μmol/L (26). Our results showed a significant relation between mild hyperhomocysteinemia (median homocysteine concentration 14.84 μmol/L) and the cerebrovascular stenosis of $= 50\%$. These results are consistent with recent studies (8, 27,28) and indicate that even mild hyperhomocysteinemia can severely impair the vascular function and thus contribute to the atherothrombotic disease. Results of the logistic regression analysis in our study did not confirm earlier studies indicating moderate hyperhomocysteinemia as an independent risk factor that may predict the severity of cerebral atherosclerosis in patients with cerebral infarction (3). Further studies on larger groups of patients are required to clear this issue.

One of the potential damaging effects of hyperhomocysteinemia in occlusive vascular disease is free radical generation leading to oxidative stress, which is closely involved in the development of atherosclerosis (12,29,30). Lipid peroxidation and antioxidant capacity in the serum of patients with atherosclerosis were examined in several studies, showing pronounced increase of lipid peroxides in patients with extensive atherosclerosis, which paralleled a decrease in antioxidant capacity (14,29,31). Our results showed that decreased values of total antioxidant status were associated with more severe forms of cerebrovascular disease, ie, stenosis of ≥50%, suggesting that total antioxidant status could be one of the independent biochemical predictor of the severity of stenosis.

Trace elements (copper and zinc) and electrolytes (total calcium and magnesium), as essential components of various enzymic and non-enzymic proteins, form a part of antioxidant system, whereas the liberated ions may be responsible for the free radical generation and progression of atherosclerosis (12-14). The results of previous reports indicate the differences in the accumulation of calcium, magnesium, zinc, and copper in the arterial wall and serum in different forms of the atherosclerotic disease (11,13,32). It was reported that serum concentrations of copper and zink were higher in obliterating atherosclerosis than in abdominal aortic aneurysm, whereas the concentrations of calcium and magnesium in both diseases were significantly lower than in the control group (11,13). Our results showed no significant differences in serum concentrations of calcium and

magnesium between patients with cerebrovascular stenosis and controls or health-associated reference values (20), whereas increased serum concentrations of copper and zinc were significantly associated with stenosis of $<50\%$.

In conclusion, our study indicates that lipoprotein(a) and total antioxidant status are most significant independent biochemical risk factors in the development of cerebrovascular stenosis. Elevated lipoprotein(a) concentration is the only risk factor significantly associated with different degree of stenosis. Low total antioxidant status value is significantly associated only with severe stenosis of $\geq 50\%$. Mild hyperhomocysteinemia seems an additional discriminating indicator of the severe cerebrovascular stenosis. These factors may be useful in early identification and recognition of patients with cerebrovascular stenosis.

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